CONFIGURATION OF THE METHOXYIMINO GROUP AND PENETRATION ABILITY OF CEFOTAXIME AND ITS STRUCTURAL ANALOGUES

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(Received for publication March 7, 1983)

Among the different mechanisms by which bacteria are resistant to β-lactam antibiotics, three are of major interest: production of β-lactamase, modifications of the target penicillin-binding proteins (PBPs) and decreased permeability. Cefotaxime (CTX) is a recently synthesized cephalosporin derivative, active against β-lactamase producing Gram-negative bacteria and possessing in the acylamino side chain a methoxyimino group in the *syn*-configuration. It has been compared for affinity to PBPs and penetration ability with its isomer possessing the same group in the *anti*-configuration and the corresponding demethoxyimino derivative.

The *anti*-isomer, although resistant to β-lactamase, is devoid of antibacterial activity (MIC for *Escherichia coli* higher than 500 μg/ml). The affinities of CTX and its analogues for the PBPs of several strains of *E. coli* have been determined *in vivo* and *in vitro* by competition experiments using intact cells and bacterial envelopes, respectively. Only minor differences in the affinity for the target PBPs were detected *in vitro*.

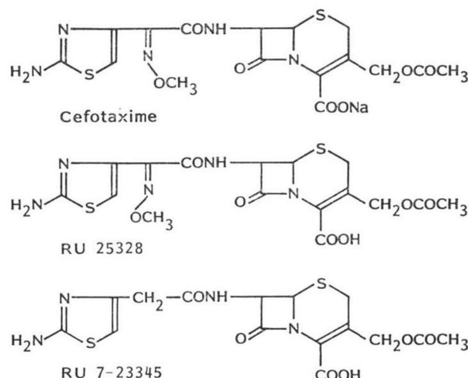
However, *in vivo* studies proved that the 50% saturating concentrations for the PBPs were more than 100-fold higher for the *anti*-isomer than for CTX.

The reported results suggest that a very simple structural modification of the CTX molecule greatly decreases the penetration ability of the antibiotic through the outer cell layers, thus dramatically affecting its antibacterial properties.

Cefotaxime (HR 756; 7-{2-(2-amino-4-thiazolyl)-2-[(*Z*)-methoxyimino]acetamido}cephalosporanic acid) is a highly active broad spectrum cephalosporin derivative[1,2] and may be considered resistant to hydrolysis by penicillinases and cephalosporinases[3]. With both types of enzymes the behavior of the isomer with the methoxyimino group in the *anti*-configuration (RU 25328) is very similar to that of cefotaxime, but this structural analogue lacks antibacterial activity.

The compound without oxime (RU 7-23345) possesses a slightly lower antibacterial activity than cefotaxime, but appears as a good substrate for the β-lactamases[3]. The structural formulas of these compounds are shown in Fig. 1.

Fig. 1. Structure of cefotaxime and its analogues.



The antibacterial activity of β-lactam antibiotics against Gram-negative bacteria depends upon: a) resistance to periplasmically located β-lactamase; b) ability to penetrate the permeability barrier of the outer membrane and c) affinity for essential penicillin-binding proteins (PBPs) located in the cytoplasmic membrane.

Because the differences in the antibacterial activity of cefotaxime and its *anti*-isomer are not related to differences in the susceptibility to β-lactamases, we have investigated which one of the other two mechanisms (decreased affinity or decreased permeability) was more relevant in determining the above described properties.

The binding affinities of the three compounds have been determined in PBPs competition experiments using both envelopes from sonicated bacteria and intact growing cells. Since the permeability barrier present in intact cells is non-operative in the *in vitro* assay system[4,5,6], a comparison between the observed affinities under the two experimental conditions is a reliable indication of the ability of the antibiotic molecules to penetrate the outer membrane[7,8].

Materials and Methods

Bacterial Strains and Growth Conditions

Escherichia coli KN 126[9], *E. coli* BUG 6[10,11], a thermosensitive division mutant, and *E. coli* SP 45[11,12], a *pbpA* temperature sensitive mutant, were used. Strains were grown in Antibiotic Medium No. 3 (Difco) at 30 and 42°C.

In Vivo Competition Binding Experiments

Exponentially growing cells (1 liter cultures) were divided into aliquots (80 ml each) and treated (15 minutes) with serial dilutions of the antibiotic to be tested (ranging from 100 to 0.78 μg/ml). Cells were immediately washed with cold phosphate buffer (50 mM; pH 7), and envelopes were prepared by differential centrifugation of sonicated cells and subsequently incubated with the radiolabelled antibiotic ([^{14}C]benzylpenicillin or [^{125}I]furazlocillin) as described below.

In Vitro Competition Binding Experiments

Envelopes were prepared from sonicated cells harvested in the exponential phase of growth (1 liter cultures). After sonication (Labsonic Mod. 1510, Braun Co., West Germany), intact cells were pelletted by centrifugation (3,000$\times g$ for 10 minutes), and the supernatant containing the membrane fraction was centrifuged at 100,000$\times g$ for 30 minutes. Envelopes were divided into ten samples and resuspended in 50 μl of 50 mM phosphate buffer. For the *in vitro* competition experiments, serial dilutions of the antibiotic to be tested (ranging from 100 to 0.78 μg/ml) were added to the envelope suspensions and incubation carried out for 15 minutes at the temperature of growth (30 or 42°C). Thereafter the radiolabelled antibiotic ([^{14}C]benzylpenicillin or [^{125}I]furazlocillin) was added at saturating concentrations, and incubation was continued for an additional 15 minutes.

In the *in vivo* competition experiments the treatment of envelopes with the unlabelled antibiotic was obviously omitted.

When [^{14}C]benzylpenicillin was used, the reaction was terminated by adding sarkosyl (2% final concentration) containing 100 mg/ml of unlabelled benzylpenicillin.

When [^{125}I]furazlocillin was used, the reaction was terminated by adding an excess of unlabelled benzylpenicillin. Membranes were then washed and resuspended in sarkosyl (2% final concentration)[14].

Separation of PBPs by SDS-PAGE and Autoradiography

After extraction (30 minutes at room temperature) and centrifugation (100,000$\times g$ for 30 minutes), the solubilized proteins were mixed with sample buffer[9] and heated at 100°C for 4 minutes. The radiolabelled PBPs were separated in a discontinuous buffer system[15] on a 7.5% acrylamide - 0.15% bisacrylamide slab gel.

When [^{125}I]furazlocillin was used, PBPs were detected after overnight exposure of the gels to X-ray films at -70°C or, when [^{14}C]benzylpenicillin was used, the gels were prepared for fluorography by the procedure of BONNER and LASKEY[16,17] with the modification that EN^{3}HANCE (New England Nuclear) was used instead of DMSO-PPO. Dried gels were exposed to X-Omat film SO-282 (Kodak) for at least 40 days.

Results

As known from *in vitro* competition binding assays, the essential PBPs for which cefotaxime has the highest affinity are PBP-3 and PBP-1A[3,8].

Under our experimental conditions, the *in vitro* 50% saturating concentrations of cefotaxime for its primary targets were almost identical for the different *E. coli* strains tested. Values were close to 0.78 μg/ml for PBP-1A and lower than 0.1 μg/ml for PBP-3, in agreement with previously published results[8] (data not shown).

When intact cells growing at 30°C were treated with serial dilutions of cefotaxime for a 15-minute pulse, the values of the 50% saturating concentrations were very similar to those obtained when the antibiotic was added to envelope suspensions (Fig. 2).

However, *in vivo*, the complete saturation of PBP-1A was not obtained even at concentrations of cefotaxime up to 100 μg/ml (Fig. 2). On the contrary, when the binding assay was performed *in vitro*, complete disappearance was obtained with 0.8 μg/ml. These results suggest that PBP-3 is the only target of effective concentrations of cefotaxime in intact growing cells.

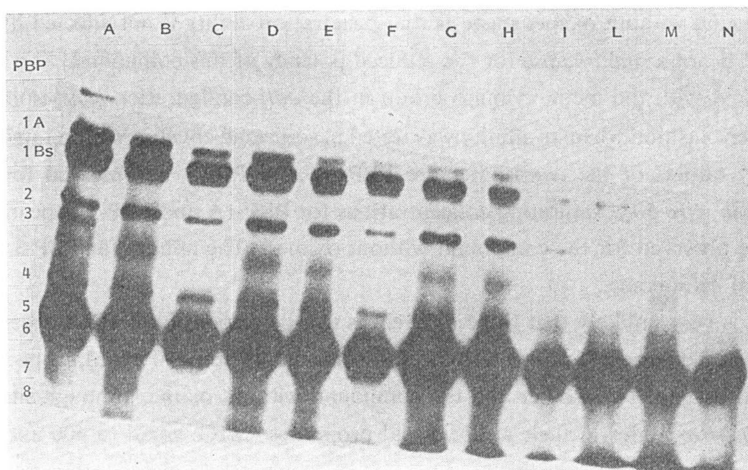
The compound without oxime (RU 7-23345) possesses the same primary targets as cefotaxime both *in vitro* (data not shown) and in intact cells (Fig. 3). The MIC values were only slightly higher than those observed for cefotaxime against the strains tested (6.25 and 1.56 μg/ml, respectively). Indeed, in both experimental situations, the affinity of RU 7-23345 for PBP-3 is somewhat lower (50% saturating concentration close to 0.2 μg/ml) than that of cefotaxime (less than 0.1 μg/ml). The two compounds show the same affinity for PBP-1A.

On the basis of these results, the poorer antibacterial activity of RU 7-23345 might be due, besides

Fig. 2. Competition of cefotaxime for [^{125}I]furazlocillin binding in intact growing cells (*in vivo* competition).

Samples (80 ml) of exponentially growing *E. coli* KN126 were treated for 15 minutes with concentrations of cefotaxime ranging from 100 (N) to 0.097 (B) μg/ml (1:1 dilutions; A=untreated control.)

Membranes were prepared by differential centrifugation of sonicated cells and incubated with [^{125}I]furazlocillin for 15 minutes (see Materials and Methods). After extraction with sarkosyl, proteins were separated by SDS-PAGE (acrylamide - bisacrylamide, 30: 0.6, 7.5% gel). Bands were detected by autoradiography.



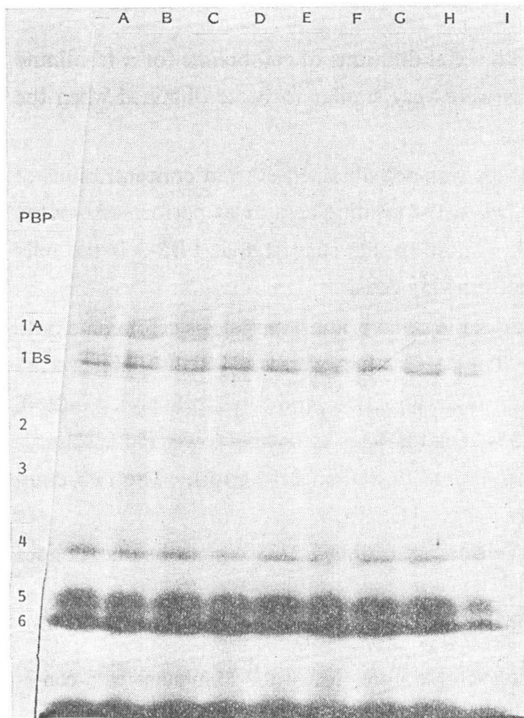


Fig. 3. Competition of the *anti*-isomer of cefotaxime for [14C]benzylpenicillin binding in membranes (*in vitro* competition).

Samples of membranes were incubated with buffer (A) or with increasing concentrations of the *anti*-isomer ranging from 0.78 (B) to 100 (I) μg/ml (1: 1 dilutions). For further details see Materials and Methods.

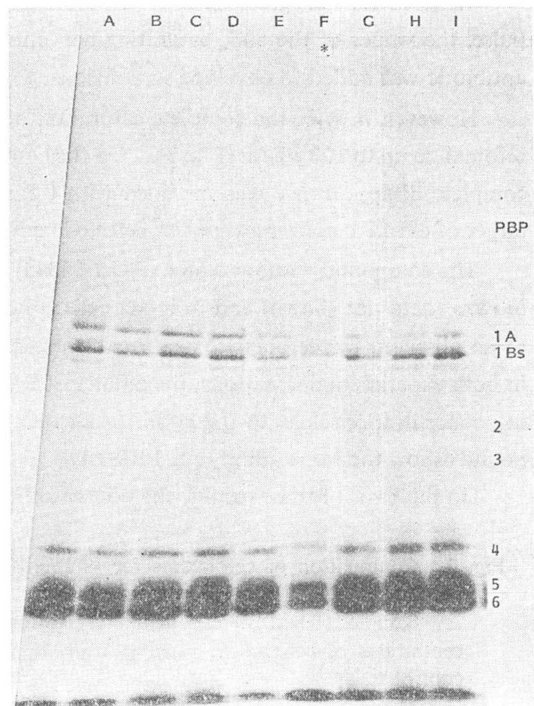


Fig. 4. Competition of the *anti*-isomer of cefotaxime for [14C]benzylpenicillin binding in intact growing cells (*in vivo* competition).

Samples (80 ml) of exponentially growing *E. coli* KN126 were treated for 15 minutes with concentrations of the *anti*-isomer ranging from 0.78 (B) to 100 (I) μg/ml (1: 1 dilutions; A=untreated control). *Failing in solubilization or in migration. For further details see Materials and Methods.

its sensitivity to the β-lactamases, to the observed decreased affinity to one of the functionally major PBPs. The similarity of the results obtained with sonicated envelopes and intact growing cells, when testing the compound without oxime, suggests that penetration ability is not affected by this structural modification and is not a major cause for the reduced potency of this compound.

The compound with the methoxyimino group in the *anti*-configuration (*anti*-isomer) behaved in a clear-cut different fashion when its affinity was tested in sonicated envelopes or in intact growing cells.

The primary targets of the *anti*-isomer are PBP-1A and PBP-3 as observed for the structural analogues. The *in vitro* 50% saturating concentrations for PBP-1A and PBP-3 appeared to be almost identical to those observed for the compound without oxime. The affinity for PBP-3 is 20～40 times lower than that of cefotaxime.

However, it is very unlikely that these differences can be responsible for the observed differences in the MICs values between cefotaxime and its *anti*-isomer (1.45 and 400 μg/ml, respectively). Moreover, it is evident that the *anti*-isomer and the compound without oxime, although showing the same binding profile *in vitro*, differ in their antibacterial properties (MICs equal to 400 and 6.25 μg/ml respectively).

More significant is the complete lack of binding ability (up to 100 μg/ml) for the target PBPs of the *anti*-isomer when tested in intact growing cells (Fig. 4). As evident from this autoradiogram, the *anti*-isomer does not compete *in vivo* for any of the PBPs although very high concentrations were used.

Discussion

A necessary condition for bacterial killing by β-lactam antibiotics is the ability to reach the PBPs in the inner membrane. In Gram-negative bacteria they have to pass through the permeability barrier represented by the outer membrane[4]. Among the several reported assays to test β-lactam permeability[4,5,7], the comparison between the *in vitro* and *in vivo* (intact cells) affinities for the PBPs has recently been shown to give direct evidence of the ability of the β-lactam antibiotics to again access to their targets (references 6, 8, 19 and M. G. BOULTON, personal communication). Indeed, when the binding is performed *in vivo i.e.* in intact growing cells in the presence of the antibiotic, the experimental condition are suitable for permeability studies, even more when non β-lactamase producing strains or β-lactamase resistant antibiotics are used.

On the contrary, *in vitro* binding assay gives information only on the affinity of the molecule for the PBPs, but not on the possible influences of permeability on the binding in the intact cells. Indeed, it is likely that the results obtained with partially purified envelopes can be affected by the orientation of the vesicles formed when cells are broken by sonication or by the French pressure cells. FUTAI[20] has demonstrated that these vesicles are inside-out. He concluded, by using different assays to determine orientation (accessibility of the impermeable ferricyanide ion to the respiratory chain, inhibition of membrane ATPase by specific antiserum, and binding of ATPase to the membrane), that the above procedures to prepare envelopes are most likely to cause inversion, with formation of inside-out vesicles.

These results can suggest a reasonable interpretation of the data reported by CURTIS *et al.*[8] who demonstrated that the binding *in vitro* of β-lactams to the PBPs is similar both in the presence or in the absence of the outer membrane. It is acceptable to assume, on the results obtained by FUTAI, that the outer membrane was inside the vesicles, thus not acting as a permeability barrier.

In our opinion, the comparison between the binding affinities in intact cells (outside-out vesicles) and in envelopes (inside-out vesicles) is a simple and reliable method to investigate penetrability of β-lactam antibiotics. All studies on new compounds should take into consideration not only the affinity profile for the different PBPs (binding *in vitro* which can be misleading), but also the ability of the antibiotic molecule to reach its target(s) in the intact bacteria.

The results obtained support the validity of our approach.

The observed decreased *in vitro* affinity for PBP-3 and PBP-1A of the *anti*-isomer (20 times lower than that of cefotaxime) cannot explain *per se* the ineffectiveness of this compound as an antibiotic. Indeed, a similar degree of affinity has been demonstrated for the compound without oxime.

Thus, if only the *in vitro* results were considered, an incorrect interpretation would have been formulated.

The binding studies with intact cells show that, in the case of the compound RU 7-23345 which maintains antibacterial activity although lacking β-lactamase resistance, the *in vivo* and *in vitro* affinity constants for the target PBPs are almost identical. On the contrary, no competition for the PBPs was detected when the *anti*-isomer of cefotaxime was tested, even at concentrations up to 100 μg/ml, in good agreement with the MIC values.

In conclusion our results support the hypothesis that the methoxyimino group in the *anti*-configuration is responsible for the lack of antibacterial activity because it seems to be associated with the inability of the molecule to come across the outer membrane barrier.

Acknowledgments

The authors wish to thank Dr. J. PERRONET (Roussel-Maestretti, Italy) for supplying the β-lactam antibiotics utilized in this study. This work has been supported by a grant from the Consiglio Nazionale delle Ricerche N° CT 810013204 to G.A.B.

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